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Review

ADVERSE EFFECTS OF COMBINED MYCOTOXINS*

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This article brings an overview of mycotoxin co-occurrence in foods in Croatia and neighbouring countries and experimental data from mycotoxin interaction studies involving *Fusarium* toxins, ochratoxin A (OTA), and aflatoxin B_1 (AFB_1). Only a few studies of combined mycotoxin toxicity have employed a mathematical/statistical design, while others have used common statistics in order to compare the effects of mycotoxin mixtures with effects of single toxins. So far, most studies have observed additive or synergistic effects, suggesting that these mixtures pose a significant threat to human and animal health.

KEY WORDS: *aflatoxin, citrinin, fumonisins, mycotoxin interactions, ochratoxin, trichothecenes, zearalenone*

Mycotoxins are the secondary metabolites of moulds and invariably contaminate food and feed all over the world. Among hundreds of known mycotoxins, aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FBs), zearalenone (ZEA), and trichothecenes stand out as the most common contaminants in a variety of food. Ingestion of these mycotoxins may cause acute toxicity or chronic disorders, depending on concentration and duration of exposure. Moreover, they are often responsible for financial losses in food production and livestock breeding. Most countries have legislation that prescribes maximum permissible concentrations of mycotoxins in certain types of food, but these regulations vary significantly between them. Croatia has harmonised its legislation on mycotoxins in foods with the European Union regulations (1).

Even though food is often contaminated with more than one mycotoxin, most studies are limited to the toxicology of a single mycotoxin. This review summarises the findings on mycotoxin co-occurrence in food in Croatia and neighbouring countries and the experimental data on their combined toxicity.

Co-occurrence of mycotoxins in food

Most of the earlier mycotoxin surveys were focused on the occurrence of single toxins in food. For example, studies conducted in Croatian and Bulgarian endemic nephropathy (EN) areas were focused on the levels of resident exposure to OTA (2, 3). Cereals are commonly contaminated with the *Fusarium* species and therefore often analysed for the presence of *Fusarium* mycotoxins such as FBs, ZEA, deoxynivalenol (DON), diacetoxiscitpenol (DAS), beauvericin (BEA), or T-2 toxin (4-6), but only a limited number of studies have paid attention to or specified the percentage of co-contaminated samples. Table 1. summarises the co-occurrence of various mycotoxins in food samples in Croatia and neighbouring countries over the last fifteen years.

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Table 1 *Co-occurrence of some mycotoxins in food from Croatia and the neighbouring countries*

Sample / Year/ Country	Mycotoxin	Contamination / %	Range / μg kg^{-1}	Co-contamination / %	Reference
Pre-harvest maize /	FB_1	100	125000 to 250000	67	81
1992-93 /	BEA	67	5000 to 10000		
Italy*					
Stored maize /	FB_1	70.8	50 to 19800	Not specified	4
Hungary	ZEA	87.5	10 to 11800		
	DON	70	70 to 21200		
	T-2	41.7	60 to 390		
Harvested maize/	FB_1	70	95 to 52400		
Hungary	ZEA	17	6 to 79		
	DON	13	50 to 118		
	T-2	39	50 to 551		
Maize /	OTA	29	0.26 to 614	Two toxins; 6	7
1996-97 /	FB_1	95	12 to 11661	Three toxins; 2	
Croatia**	BEA	11	13 to 1864		
Grains and feeds /	T-2	16.8	100 to 700	Not specified	6
1998-2004 /	DAS	27.6	100 to 500		
Croatia***	DON	41.2	100 to 3440		
Cereals /	OTA	35	<0.5 to 140	22	8
1999 /	CTN	9.4	<5 to 420		
Bulgaria**					
Maize /	OTA	33	0.73 to 2.54	Two toxins; 55	82
2002 /	FB_1	100	196.8 to 1377.6	Three toxins; 37	
Croatia	FB_2	13	68.4 to 3084		
Foodstuffs /	FB_1	26	10 to 2870	Not specified	5
2002 /	FB_2	35	10 to 790		
Italy	DON	84	7 to 930		
Spices /	AFB_1	21	6.1 to 15.7	Not specified	83
2004 /	OTA	36	10.6 to 66.2		
Hungary					
Total diet	OTA	34	0.29 to 2.25	10	9
Serbia**	CTN	16			
Maize, feed /	AFs	24.3	2.3 to 10.3	Two toxins;	15
2007 /	OTA	16.2	2.5 to 31.7	4.2 to 54	
Croatia**	ZEA	92	12.5 to 1182	Three toxins;	
	$FB_1 + FB_2 + FB_3$	27	200 to 20700	4.2 to 7.6	
Pasta /	AFB_1	Not detected		Not specified	84
Italy	OTA	96.3	0.2 to 0.52		
	DON	81.5	35.1 to 450.0		

** Only six maize samples were analysed*
*** Samples were taken in the EN area*
**** A total of 465 samples were analysed*

FB_1 is the most frequent maize contaminant which possesses several toxic properties (Table 2). It occurs in significantly higher concentrations than any other *Fusarium* toxin. It is often accompanied in maize by ZEA, DON, or OTA. Due to its nephrotoxicity, special attention has been paid to its co-occurrence with OTA as well as to co-occurrence of OTA and citrinin (CTN), particularly in the EN areas (2), as these areas report higher crop contamination with OTA and FB_1 or OTA and CTN

Table 2 *Mechanism of action and toxic properties of some mycotoxins, frequently found as food and feed contaminants.*

Mycotoxins	Primary events at the cellular level	Toxic properties	References
Aflatoxin B_1	Metabolic activation → AFB1-8,9-epoxide → modification of major cell macromolecules	Hepatotoxic, immunosuppressive, carcinogenic (group 1, IARC), teratogenic, mutagenic	66, 67
Beauvericin	Complex with essential cations (Ca^{2+}, Na^+, K^+) → inhibition of cation-selective channels in lipid membranes, inhibitor of cholesterol acyltransferase	Antimicrobial, insecticidal cytotoxic, ionophoric action	85
Citrinin	Affects mitochondrial permeability transition, calcium flux, and cytochrome c release from mitochondria, inhibits macromolecule biosynthesis → cell death	Nephrotoxic, hepatotoxic genotoxic (group 3, IARC), teratogenic, immunotoxic	86-88
Deoxynivalenol	Inhibition of protein synthesis → cell death, disruption of cytokine regulation	Causes nausea, food refusal, vomiting, diarrhoea, immunotoxic, IARC group 3	89-91
Diacetoxyscirpenol and T-toxin	Inhibition of protein synthesis → cell death T-2 → erythrocyte lysis, induction of lipid peroxidation, apoptosis, inhibition of mitochondrial electron transport	Alimentary toxic aleukia (inflammation of the skin, vomiting, damage to hematopoietic tissues, haemorrhagia), IARC group 3	89-92
Fumonisin B_1	Inhibition of ceramide synthase → changes in sphingolipid metabolism, → protein kinase activity, oxidative stress → oxidative damage of cell macromoleculs	Equine leukoencephalomalacia, porcine pulmonary oedema, hepatotoxic, nephrotoxic immunosuppressive, carcinogenic (group 2B, IARC)	89-91, 93
Ochratoxin A	Competition with phenylalanine and inhibition of Phe-dependent enzymes, inhibition of protein and DNA synthesis, mitohondrial transport system and transport of organic anions and cations, oxidative stress, DNA damage, affects glucose metabolism and Ca^{2+} homeostasis,	Nephrotoxic, neurotoxic hepatotoxic, affects blood coagulation, immunotoxic carcinogenic (group 2B, IARC) teratogenic	9, 50, 94
Zearalenone	Resembles 17b-oestradiol → binds to oestrogen receptors in mammalian target cells	Disruption of hormonal control, IARC group 3	89, 91

than non-EN villages in Bulgaria, Croatia, and Serbia (7-9). Until recently it was thought that FBs were produced only by the *Fusarium* species that contaminate maize and that their occurrence in foods was limited to that substrate. However, black Aspergilli, including *A. niger* and *A. awamori*, which contaminate substrates with a high sugar content such as dried fruits, are able to produce FB_{1-4}, 3-epi-FB_3, 3-epi-FB_4, iso-FB_1, and two iso-$FB_{2,3}$ forms (10). Therefore, FBs and OTA could co-

contaminate these substrates as well as grapes, and both toxins could be expected in red wine (11). In addition, *A. niger* strains, which have extensively been used in biotechnology and fungal mycelium from fermentation as animal feed, can produce both toxins (12). Taking into account these investigations, FBs and OTA could be expected in various foods and not only in maize.

Mould production of mycotoxins in food is influenced by a number of factors such as temperature, water activity, substrate composition, mould physiology, or interactions with other microbes (13). All these factors are directly linked to climate change. For example, the optimal temperature for the formation of AFs is 33 °C, and these toxins are far more common in the hot tropical and subtropical regions. With current climate changes, however, AFs may soon become more common in the temperate areas of Europe as well (14). The case in point are recent reports from Croatia; AFs were found in relatively many cereal samples (24.3 %) and feed in respect to earlier studies (15, 16). The increasing incidence of AF producers (*A. flavus*) in moderate climate zones may in turn reduce the number of *Penicillium* OTA producers, as OTA production requires lower temperatures (14). The optimal temperature for the formation of *Fusarium* toxins is between 20 °C and 30 °C, and one might expect a reduction in their incidence with temperature rising above 30 °C. However, *F. verticillioides*, a producer of the FBs which frequently infect maize in Southern Europe, has been associated with dry weather during grain fill and late season rains (17). Therefore, the production of FBs will be favoured by the climate change (18). Mild temperature and rain during maize growth makes the plant susceptible to infection by *F. graminearum*, a producer of DON and ZEA. The production of ZEA is also favoured by temperature fluctuations (19). In addition, cereal infections by *Fusarium* species are spread by insects whose number is also increasing due to the climate change (14, 18). All this suggests that increased occurrence of AFs in food and co-occurrence with *Fusarium* toxins may become a food safety problem in this part of Europe in the near future.

Mycotoxin interactions at the cellular level: cell models and statistics

Combined toxicity is very hard to predict because it is influenced by several factors, including chemistry and mechanism of action, toxicodinamics and toxicokinetics, experimental design and endpoints of the study as well as statistical aspects (Figure 1). The best approach to studying combined toxicity is to find out how a single mycotoxin acts at the cellular level and how it interacts with another mycotoxin (20). Riley (21) has indicated where mycotoxins can interfere in the cascade of cell machinery and thus affect cellular function. For example, deregulation of calcium ions can at the same time affect mitochondrial function and activate cell signal molecules or endonucleases, which in turn can lead to the inhibition of protein synthesis or to DNA or cell membrane damage, and finally to cell death. This means that multiple mycotoxins can affect certain targets and initiate more than one event in the cell machinery leading to extremely complicated cell response (20, 21). Table 2 shows the toxic properties and toxicity mechanisms of some *Fusarium* toxins, AFs, OTA, and CTN, which frequently contaminate food in Croatia and the neighbouring countries.

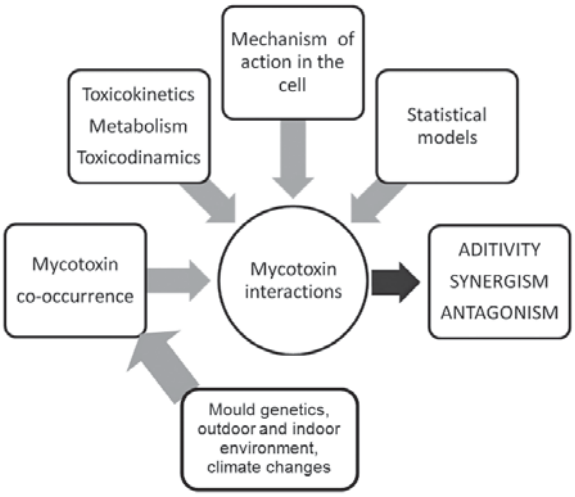

Figure 1 *Influence of various factors on mycotoxin interactions outcome in experimental model*

Over the last few years researchers have used more and more cell cultures to study the mechanisms of mycotoxin action (cell-specific function toxicity) and to predict the outcome of mycotoxin mixtures. Cell cultures have many limitations such as immortalisation, limited survival, metabolic imbalance, or absence of tissue communication (22). Nevertheless, these *in vitro* systems are particularly useful for studying the interaction between low-molecular-weight compounds such as mycotoxins and their mixtures, and cell macromolecules. For toxicity screening, Gutleb et al. (23) recommend that several cell lines are used, as they differ in sensitivity. For example, some mammalian

cells are sensitive to fumonisin cytotoxicity. Furthermore, different cell lines can answer specific questions related to mycotoxin kinetics, active transport, metabolism, or biotransformation (22). For example, Caco-2 cell (human epithelial colorectal adenocarcinoma) layers are useful for studying absorption in the small intestine, because these cells morphologically and functionally resemble enterocytes in the small intestine (24); Hep G2 cells (human hepatocellular carcinoma) are useful for studies of liver metabolism and toxicity of xenobiotics, while artificially increased expression of specific P450 cytochromes makes them sensitive to promutagens such as aflatoxins or related mycotoxins (25). Human lung adenocarcinoma (A549) cells express phase I and phase II enzymes involved in detoxification or bioactivation of respiratory toxins (e. g. sterigmatocystin-STC and *Stachybotrys* toxins) and are morphologically and functionally consistent with type II pulmonary epithelial cells *in vivo* (26). A parallelogram approach makes it possible to compare data derived from animal cell studies and animal-derived data as well human-derived *in vitro* systems and animal cell systems (22).

Choosing the right cell model and carefully planning experimental design for studying mycotoxin interactions are essential for accurate mathematical and statistical analysis. So far, only a few mathematical/statistical models have been applied for mycotoxin mixture experiments, including the central composite design, full factorial design (27, 28), ray design (29), isobolographic analyses (30, 31), and unpaired *t*-test for the comparison between expected and measured toxicity of a mycotoxin mixture (28, 32-37). The aim of these mathematical designs is to predict combined mycotoxin effects based on the comparison between the observed and expected effects of a mycotoxin mixture. The ray design provides constant mixture ratios considering the concentration-response relationship. This design compares responses to concentration addition of individual toxins and can be related to the comparison between the expected and measured toxicity of combined mycotoxins analysed by the *t*-test. An alternative is the composite design (*n* x *n*), where particular mixture compositions are selected and analysed using the full factorial design to detect interactions at various mixture ratios. The isobolographic analysis is also based on the concentration-response relationship of individual and combined mycotoxins and provides a combination index as a quantitative measure of the degree of interaction between mycotoxins in the mixture. These mathematical/statistical designs were employed for studying interactions between *Fusarium* mycotoxins (27, 30, 31), CTN, and OTA (28, 29, 32, 37), then OTA and FB_1 or BEA (33-36), and between AFB_1 and OTA or FB_1 (38, 39). The results of these studies will be discussed in the following sections.

Interactions between Fusarium *mycotoxins*

Studies of trichothecenes are mainly focused on their cytotoxic and immunomodulatory effects. Speijers and Spejiers (20) have reviewed studies of interactions between trichothecenes (DON, DAS, T-2, nivalenol-NIV and moniliformin-MON) as well as between ZEA and FB_1, including the one by Tajima et al. (27) and Thuvander et al. (40). In the latter study trichothecenes DON, DAS, T-2, and NIV inhibited both the proliferation of human lymphocytes and immunoglobulin (Ig) production *in vitro*. Combinations of NIV and other trichothecenes resulted in additive toxicity, while DON combined with T-2 or DAS showed a slight antagonism. Tajima et al. (27) investigated the inhibition of DNA synthesis in the mouse fibroblast cell line L-929 treated with NIV, DON, T-2, ZEA, and FB_1 alone and in two-toxin combinations. Single toxins showed concentration-dependent inhibitory effects in the following order: T-2>DON and NIV>ZEA, while FB_1 induced cell proliferation. Combinations produced additive effects at lower concentrations of individual toxins, while no additive effect was detected at high concentrations. ZEA and FB_1 and NIV and T-2 showed synergistic effects in the second, "screening stage" of the experiment. These particular mixtures were then separately studied using the full factorial design, which detected synergism between NIV and T-2 , but did not confirm the one between ZEA and FB_1. The authors concluded that the effect of mycotoxin mixture cannot be predicted solely on the basis of the effect of the individual toxins.

Several studies (41-43) found epithelial porcine kidney (PK15) cells highly sensitive to relatively low concentrations of trichothecenes and sphinganine analogues and useful in studying the toxicity of combined *Fusarium* mycotoxins (fusarochromanone-FUCH, NIV, 4-ac-NIV, 15-acNIV, scirpentriol-SCIRP, and DAS) found in rice culture extracts of *F. equiseti*. The relationship between single toxins was determined by the factor analysis based on correlations, while the relationship between the toxicity of culture extracts and contribution of toxins was studied using the

multiple regression analysis. The cytotoxicity of extracts was mainly attributed to the combination of FUCH and 15-ac-NIV, while SCIRP or DAS were also implicated in this mixture (42).

In a study by Luongo et al. (44), FB_1 and α-ZEA alone exerted different immunomodulatory effects on human lymphoblastoid Jurkat T cells, but in combination FB_1 induced concentration-dependent proliferation while α-ZEA was cytotoxic. The isobole analysis showed that this mixture potentiated α-ZEA cytotoxicity. In addition, this mycotoxin combination significantly reduced interferon γ (INFγ) mRNA expression compared to α-ZEA alone. Using the isobole analysis Ruiz et al. (30, 31) showed that a combination of BEA and DON or T-2, as well as the three-toxin combination had antagonistic effects in mammalian kidney epithelial Vero cells. However, these mixtures exerted both synergism (BEA+T-2 and BEA+DON+T-2) and antagonism (DON+BEA and DON+T-2) in Chinese hamster ovary CHO-K1 cells, depending on the time of exposure. Dombrink-Kurtzman (45) reported additive apoptotic effect of FB_1+BEA on turkey peripheral blood lymphocytes. In other words, the same toxins interact differently in different cells.

Only a few studies addressed combined effects of *Fusarium* toxins in animals (46, 47). A combination of DON and MON as well as of FB_1 and DAS or OTA exerted additive or less than additive toxicity. Similarly, Müller et al. (48) did not observe significant interactions between low-dose FB_1, DON, and T-2 with OTA in weaning piglets. In contrast, chronic ingestion of single and combined FB_1 and DON caused significant morphological and immunological changes in the intestine of piglets studied by Bracarense et al. (49). Synergy was observed in the number of goblet cells and eosinophils in the ileum, additive interactions in the expression of IL-10, TNF-α, and adherent proteins, less-than-additive interactions in the expression of INF-γ and lesion scores, and antagonistic effects in goblet cells, plasma cells, eosinophils, and lymphocytes of the jejunum and in the expression of IL-1β and IL-6. This study suggests that ingestion of low doses of these toxins may predispose animals to infections by enteric pathogens through an interactive alteration of the intestine. The authors have based these conclusions on the statistical analysis of combined treatment versus control and single-toxin treatment, but their results have not been verified by mathematical or statistical models that compare theoretical and measured effects of mycotoxin mixtures.

Mycotoxin interaction studies involving OTA

OTA is one of the most studied mycotoxins due to its presence in a variety of food commodities (maize, wheat, beans, grapes, and wine) as well as due to its implication in the development of EN in humans (50, 51). This mycotoxin is often found in food together with FBs, ZEA, or CTN (Table 1).

OTA and CTN were studied for nephrotoxicity separately and in combination. In combination, they were dominantly synergistic both in vivo and in vitro (see review 20). When CTN or patulin (PAT) were combined with OTA or OTB in porcine kidney LLC-PK1 cells, these binary mixtures always resulted in higher toxicity that was confirmed as synergism by the full factorial analysis. The toxicity of these mixtures was not as pronounced in human proximal tubule-derived IHKE cells as in LLC-PK1 cells (28, 52). In contrast, CTN had an antagonistic effect on OTA-induced caspase 3-activation in IHKE cells, which might be explained by a decreased uptake of OTA into the cells when both toxins were applied at the same time (32). Concurrent application of OTA and CTN in PK15 cells resulted in apoptotic and necrotic synergism, while genotoxicity, determined by the micronucleus test, was either additive or antagonistic, depending on mycotoxin concentrations in the mixture. Co-treatment of PK15 cells with calcium chelator BAPTA-AM plus OTA and/or CTN showed that calcium played a significant role in both DNA damage and cell death (37). In green monkey kidney Vero cells a combination of OTA and CTN significantly increased cytotoxicity and DNA fragmentation while in mouse bone marrow cells, chromosome aberrations were observed. Compared to the effects of single toxins, the effect was synergistic (53). Genotoxic synergism was also observed in rats fed with OTA and CTN and in human kidney (HK2) cells (54, 55). In the kidneys of animals receiving both toxins, the amount of DNA-OTA adducts (dG-OTA) increased ten times. In HK2 cells it increased two times. Co-exposure to CTN and OTA significantly increased cyclooxygenase (COX2) and lipooxygenase (LOX) expression, which corresponded to the increase in dG-OTA adduct formation. This study supports previous findings that OTA-mediated DNA adducts are under the control of biotransformation enzymes such as CYP450 1B1, 2C9, COX, and LOX (9, 50). Before these studies, it was believed that OTA poorly metabolised and did

not form reactive intermediates capable of interacting with DNA (50). However, mass spectrometry studies have shown that OTA-derived quinine/hydroquinone redox couple is involved in the generation of DNA adducts (56, 57). This genotoxic synergism might play a role in the development of EN and related chronic kidney diseases and carcinomas.

Beside the nephrotoxic potential, OTA in combination with CTN, PAT, and gliotoxin was also studied for immunotoxic effects in human peripheral blood mononuclear cells (29). The ray design showed that lower concentrations of these toxins in mixtures caused stronger inhibition of interferon-g release from T-cells and natural killer cells.

The last decade has seen intensive research of the combined effects of OTA and FB_1 (33-36, 58-65). In most cases, these toxins interacted in a synergistic or at least additive manner. Cytotoxic synergism was observed in rat brain glioma C6 cells, human intestinal Caco-2 cells, and Vero cells. These cells were exposed to low FB_1 and high OTA concentrations, which showed cytotoxicity above additive effects in respect to the individual toxins (58). Subcytotoxic concentrations of OTA and FB_1 or OTA and BEA in the mixture additively increased lipid peroxidation and decreased the level of glutathione in PK15 cells. At the same time, these mixtures synergistically induced caspase-3 (33, 34). All three mycotoxins applied at subcytotoxic concentrations induced DNA damage in PK15 cells (measured by the micronucelus and/or comet assay), and their combinations mostly produced an additive genotoxic effect (35, 36). The ionophoric activity of BEA is probably responsible for changes in membrane permeability, facilitating the penetration of FB_1 and OTA into the cell. In a study by Domijan et al. (59) both the Fpg-modified and standard alkaline comet assay showed that OTA+FB_1 synergistically induced DNA damage in the kidneys of male Wistar rats, indicating that beside oxidative stress some other mechanism is also involved in their genotoxic effect. A recent study by Hadjeba-Medjdoub et al. (60) has shown that FB_1 promotes OTA-specific DNA adducts, including C-C8dG OTA adduct and OTHQ-related adduct. These specific adducts are found in human urothelial tumours in the EN areas.

In pig studies (61-63), a mixture of OTA and FB_1 caused stronger lesions in the kidneys, more pronounced changes in biochemical parameters, and disturbances in humoral immune response in doses that corresponded to those found in cases of porcine nephropathy in Bulgaria and South Africa. These findings suggested that mixtures of OTA and FB_1 were involved in the aetiology of the disease.

Stoev et al. (64) found that beside FB_1 and CTN, penicillic acid (PA) also co-occurred with OTA in swine feed. PA inhibited carboxypeptidase, the enzyme involved in detoxification of OTA, which may have enhanced OTA toxicity. This and another study by the same authors (65) showed that a combination of OTA+PA produced synergistic toxicity, expressed as degenerative changes of internal organs in chickens and pigs. Experimental administration of these toxins provoked degeneration that corresponded to porcine nephropathy, which suggested that the disease may be owed to a combined action of two or more mycotoxins.

Mycotoxin interaction studies involving aflatoxins

Toxic properties of AFs have well been documented over the past five decades in both *in vivo* and *in vitro* models (66-69). Of the major AFs (AFB_1, AFB_2, AFG_1, AFG_2), AFB_1 is the most prevalent and most toxic. Its toxicity and carcinogenicity are linked to the metabolic conversion by the cytochrome p450 to a highly reactive AFB1-8,9-epoxide, which binds to the DNA, RNA, and proteins. AFB_1 co-occurred with FB_1 in a high-incidence area of human primary hepatocellular carcinoma in Haimen and Guangxi (Republic of China), suggesting that the mixture may be involved in the development of the disease (68, 69). This is why this combination has received the greatest attention over the last decade. Studies on growing barrows and turkey poults fed with this binary mixture resulted in additive to synergistic effects in the barrows and less-than-additive to additive effects in the poults as compared to each mycotoxin alone (70, 71). Pozzi et al. (72) reported a synergistic effect on the reduction of body weight gain in male Wistar rats receiving oral AFB_1 and FB_1. Galderblom et al. (73) found that in the liver of male Fischer rats, FB_1 exerted between 200 and 400 times lower cancer initiating potential than AFB_1. However, administration of FB_1 three weeks after AFB_1 resulted in a synergistic increase in hepatocyte nodules and foci. In contrast, Carlson et al. (74) reported no cancer-initiating properties of FB_1 in rainbow trout but enhanced tumour formation, which points to its cancer-promoting activity. Gelderblom et al. (75) have suggested that the inhibition of cell proliferation is an important mechanism for the cancer-promoting activity of FB_1. These studies showed that AFB_1 and FB_1 interacted

synergistically in both cancer initiation and promotion, depending on intake conditions.

In a study by Theumer et al. (76), subchronic doses of the AFB_1+FB_1 mixture induced more pronounced apoptosis in the liver of male Wistar rats than either toxin alone. In addition, this combination provoked a significant increase in Sa and Sa/So ratio in the kidney and liver compared to FB_1 alone, indicating that AFB_1 enhanced FB_1-induced impairments of sphingolipid metabolism. In another study by Theumer et al. (77) the toxins, applied alone or in combination, affected the immune response of rats differently. Spleen mononuclear cells (SMC) of rats fed with AFB_1+FB_1 produced higher levels of IL-4 and lower levels of IL-10 compared to the SMC of animals fed with AFB_1 alone. This suggests that subchronic doses AFB_1 and FB_1 may produce an imbalance in TH_1-TH_2 cellular subpopulations. Furthermore, peritoneal macrophages in rats exposed to AFB_1 alone released less H_2O_2 than in those exposed to a toxin mixture. The mechanism of these actions is still unknown. In yet another study, Theumer et al. (78) used the alkaline comet assay and the micronucleus test to measure DNA damage in SMC in Wistar rats exposed to subchronic doses of AFB_1, FB_1, and their mixture. All three induced a significant damage. The parameters of oxidative stress (malondialdehyde, catalase, and superoxide dismutase) were also elevated, supporting the assumption that FB_1 causes oxidative stress. DNA damage was more pronounced in animals fed with AFB_1 or a mixture of AFB_1 and FB_1 than in rats exposed to FB_1 alone, indicating that the DNA lesions were produced mainly by AFB_1.

In a study by McKean et al. (39), a combination of AFB_1 at the ¾ LD_{50} and FB_1 at a dose that did not provoke acute toxicity resulted in 100 % mortality in F-344 rats, which could be considered a synergistic effect. Using an interactive index the authors confirmed that the toxic effect was at the margin of synergism. Mosquitofish (*Gambusia affinis*) and human bronchial epithelial BEAS-2B cells showed similar findings, while in HepG2 cells this binary mixture produced antagonistic effect. In another study (79), McKean et al. used the same experimental and mathematical model to establish the combined toxicity of AFB_1 and T-2. The two toxins interacted mostly in an additive manner, while a synergistic interaction was noted in BEAS-2B cells.

In chicks fed with a mixture of AFB_1 and OTA, Huff et al. (38) established a synergistic effect on growth inhibition and mortality. The primary effect of this interaction was nephrotoxicity but not hepatotoxicity. Recently, this combination was tested in cultured monkey kidney Vero cells (94). It significantly decreased cell viability, increased DNA fragmentation and p53 activation, and decreased the expression of the anti-apoptotic factor bcl-2. The interaction between these toxins was additive.

CONCLUSIONS

Through food humans and animals are constantly exposed to combinations of mycotoxins in a variety of concentrations. Current climate changes have influenced the distribution of mycotoxin producers and consequently the presence of particular mycotoxins as food contaminants (e.g. AFs). Exposure to high mycotoxin concentrations often provokes acute symptoms, which are seldom recognised as mycotoxicoses. Chronic intake of sub-toxic mycotoxin concentrations might alter various events at the cellular level, leading to biochemical and immunological impairments and possibly cancer. The relationship between chronic conditions and exposure to mycotoxins is even more difficult to recognise. In general, most of the mycotoxin mixture studies have observed additive or synergistic interactions, calling our attention to the significant threat to human and animal health. One of the preventive measures should be to revise current legislation on exposure limits, taking into account these new data. Future research may expand to include other relevant mycotoxins or combinations.

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Sažetak

ŠTETNI UČINCI KOMBINIRANIH MIKOTOKSINA

U članku je prikazan pregled mikotoksinske kokontaminacije hrane u Hrvatskoj i susjednim zemljama te njihovih kombiniranih toksičnih učinaka na različitim eksperimentalnim modelima. Pritom su obuhvaćene studije interakcija koje uključuju fuzarijske mikotoksine, okratoksin A (OTA) i aflatoksin B_1 (AFB_1). Nekoliko je takvih istraživanja napravljeno na temelju matematičko-statističkog modela, dok je većina studija primijenila jednostavnu statističku analizu koja omogućava usporedbu učinaka kombiniranih mikotoksina u odnosu na učinke pojedinačnih. Općenito, većina dosadašnjih studija pokazuje da kombinacije mikotoksina u biološkom sustavu imaju sinergistički ili barem aditivni učinak, što znači da su velik rizik za zdravlje ljudi i životinja.

KLJUČNE RIJEČI: *aflatoksin, citrinin, fumonizini, interakcije mikotoksina, okratoksin, trihoteceni, zearalenon*

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